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Growth arrest by induction of p53 in DNA damaged keratinocytes is bypassed by human papillomavirus 16 E7

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ABSTRACT **Cellular tumor suppressors p53 and Rb play an important role in controlling cell proliferation. Inactivation of these tumor suppressor proteins can occur by gene mutation or by association with oncoproteins from the small DNA tumor viruses. One function of p53 is in regulating cell cycle checkpoint control after DNA damage. To dissect the pathways by which p53 and Rb may act, the *E6* and *E7* oncogenes of human papillomavirus (HPV) types 6 and 16 were introduced into primary human epithelial cells by retroviral transfer vector, and cells were assayed for growth arrest after DNA damage induced by actinomycin D. The *E6* or *E7* oncogenes from the low-risk HPV6 had no affect on growth arrest, p53 protein levels increased, Rb protein levels decreased, and Rb was predominantly in the hypophosphorylated state similar to vector-infected cells. Either the *E6* or the *E7* oncogene from the high-risk HPV16 abrogated growth arrest. Cells expressing HPV16 E6 (16E6) were severely reduced in p53 protein levels that did not increase detectably after DNA damage, Rb protein levels did not decrease, and hyperphosphorylated Rb was present. After DNA damage in cells expressing 16E7 p53 levels increased, and Rb protein levels decreased; however, Rb was predominantly in the hyperphosphorylated state. Even though p53 protein levels increased in response to DNA damage in cells expressing 16E7, G_1 growth arrest was bypassed. This suggests that the circuitry controlling the growth arrest signal after DNA damage may be interconnected between the p53 and Rb pathways.**

The link between tumor suppressor proteins and cell cycle control is not clear. The subsequent inactivation of the wild-type p53 or *RB1* gene in patients that carry mutant and wild-type alleles in the germ line is responsible for the inherited cancer susceptibility syndromes in humans Li-Fraumeni and retinoblastoma, respectively (1). Mice engineered to be lacking the p53 gene developed normally but had a predisposition for tumor formation, indicating that cell divisions and complete normal development occurred in the absence of p53 (2). Mice generated without Rb developed up to days 12–14 in embryogenesis, suggesting that, as in the case of p53, cell divisions can occur normally in the absence of Rb; however, Rb apparently was required for some cells of the neuronal and erythropoietic lineages to differentiate normally (3–5).

The mechanism by which Rb is able to control cell proliferation at the cell cycle level is thought to involve binding and release of E2F, a transcriptional activator, due to cyclic phosphorylation and dephosphorylation events of Rb during the cell cycle (reviewed in ref. 6). Rb is underphosphorylated early in G_1, binding to E2F, and becomes heavily phosphorylated at the transition of G_1 to S, thereby releasing free E2F. E2F binding sites on the dihydrofolate reductase promoter are responsible for up-regulation of transcription at the G_1/S boundary for this S-phase gene (7). Cyclin/cdk complexes have been implicated in controlling the phosphorylation events of Rb and the ability to relieve Rb suppression of growth (8, 9), thus connecting a tumor suppressor gene with the cell cycle.

One way that p53 may act as a tumor suppressor is in controlling a G_1 checkpoint after DNA damage (10). Upon DNA damage, cells growth arrest in G_1 and G_2. p53 has been shown to mediate G_1 growth arrest in that cells with mutated or no p53 do not growth arrest in G_1 after DNA damage (11). G_2 arrest is not affected in cells with mutated p53. Cells with no p53 can undergo gene amplification (12), further suggesting a role for p53 in maintaining genomic stability.

The inactivation of cellular tumor suppressor proteins p53 and Rb by association with viral oncoproteins is a mechanism shared among the small DNA tumor viruses simian virus 40 (SV40) (13, 14), adenovirus (15, 16), and human papillomavirus (HPV). HPV has been identified as an etiologic agent in anogenital cancers (17). Only a particular subset of HPVs, such as types 16 and 18, that infect the anogenital tract are associated with high risk of malignant conversion (18). Other types, such as 6 and 11, are associated with benign genital warts and are at low risk for malignant conversion. The introduction of *E6* and *E7* genes from high-risk types is sufficient for efficient immortalization of primary human keratinocytes (19–21). *E7* binds to the tumor suppressor Rb (22) and related proteins such as p107 and p130 (23) and is the dominant oncogene since rare colonies can become immortalized that harbor only *E7* (21, 24). The E6 protein binds to the cellular tumor suppressor p53 (25) and targets p53 for degradation via the ubiquitin proteolysis pathway (26). In addition, it has been shown that HPV16 E6 (16E6) is able to abrogate growth arrest induced by DNA damage, presumably due to degradation of the p53 protein mediated by 16E6 (27).

In this report we show evidence that the *16E6* or *16E7* genes, but not the *6E6* or *6E7* genes, were able to abrogate cell cycle checkpoint control. In cells expressing 16E7, p53 protein levels increased upon actinomycin D treatment by a similar amount to cells expressing 6E6 or 6E7, yet the cells circumvented G_1 growth arrest. The underphosphorylated form of Rb was dominant in cells that did undergo growth arrest, but phosphorylated forms were dominant in cells expressing 16E6 or 16E7 after treatment.

MATERIALS AND METHODS

Cells. Primary human keratinocytes were derived from neonatal foreskin and grown in GIBCO K-SFM supplemented with penicillin/streptomycin. Cells were infected with amphotropic retroviruses containing vector (LXSN), HPV6 or -16 genes for E6 or E7 (LXSN-6E6, -6E7, -16E6, -16E7, and -16E6E7) and have been described (28). Pooled populations were used one to three passages after G418 selection.

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Abbreviations: SV40, simian virus 40; HPV, human papillomavirus; FITC, fluorescein isothiocyanate; PI, propidium iodide; HFE, human foreskin epithelial cell.

Radioimmunoprecipitations. Cells were labeled for 2 h with 200 μCi of [^{35}S]cysteine and 100 μCi of ^{35}S express label (Amersham) per 100-mm plate (1 Ci = 37 GBq). Rabbit polyclonal antisera generated against HPV6 and -16 E6 and E7 bacterial fusion proteins (29) was used to immunoprecipitate the HPV proteins. Radioimmunoprecipitations were done as described (30).

Growth Arrest. DNA damage was induced by treating the cells for 24 h with 0.5 nM actinomycin D (31, 32). Cells were prepared for flow cytometry analysis as described (33) with slight modifications. Briefly, cells were pulsed for 4 h with 0.01 mM bromodeoxyuridine (BrdUrd). After trypsin treatment cells were fixed in 66% cold ethanol. Nuclei were isolated by pepsin treatment. DNA was denatured with 2 M HCl and stained with fluorescein isothiocyanate (FITC)-conjugated anti-BrdUrd (Becton Dickinson) and propidium iodide (PI). Nuclei were analyzed by FACScan (Becton Dickinson); 10,000 events were analyzed. Doublets were discriminated by gating on PI staining of area vs. width. ReproMan (TrueFacts Software, Seattle) was used to display and quantitate the two parameter plot of FITC (DNA incorporation) vs. PI (total DNA) staining.

Western Blots. Total cell lysates were run on SDS/polyacrylamide gel and transferred to poly(vinylidene difluoride) membrane. Western blots were done using a 1:1000 dilution of anti-p53 Ab6 (Oncogene Science) or anti-Rb (PharMingen) followed by a 1:50,000 dilution of secondary anti-mouse IgG horseradish peroxidase conjugate. Detection was by chemiluminescence. Relative levels of target protein were determined by integrated optical density using Optimus software (Bioscan, Edmonds, WA).

RESULTS

Primary human foreskin epithelial cells (HFEs) were infected with retroviruses expressing the *E6* and *E7* oncogenes from HPV6 and -16, selected for neomycin resistance, and immediately assayed for growth arrest after DNA damage induced by actinomycin D. Therefore, the effects of the oncogenes on growth arrest after DNA damage were assessed in the absence of further cellular events that might have occurred during prolonged passage or crisis in culture. At low concentrations, actinomycin D induces DNA breaks similar to ionizing radiation by inhibiting cellular topoisomerases (31, 32) and has been shown to cause G_1 arrest (10, 34) and induce p53 protein levels (10). Effects on cell metabolism other than DNA damage that actinomycin D may exert are limited by the low dose, which is 10,000-fold less than the concentration used to inhibit RNA synthesis.

Expression of HPV Oncoproteins. Fig. 1 demonstrates that the retroviruses used in these assays expressed the papillomavirus proteins in HFEs immediately after selection. Cells were labeled with [^{35}S]methionine and [^{35}S]cysteine at one passage postselection. Radioimmunoprecipitations were done by using antibodies directed against bacterially expressed fusion proteins (29). Each of the papillomavirus proteins was immunoprecipitated from the cells infected with retroviruses using the cognate antibodies.

Growth Arrest Assays. To assay for the effects of the HPV oncogenes on the ability of cells to growth arrest, cells were exposed to actinomycin D for 24 h and then pulsed with BrdUrd for 4 h. Cells were stained with FITC-conjugated anti-BrdUrd (FITC) to detect DNA incorporation during the pulse and with PI to stain for total DNA content. A two-parameter plot, FITC vs. PI staining, displays the cells in S phase as an arc above the G_0/G_1 and G_2/M populations. A G_1 block can be seen in the vector (LXSN)-infected cells that were treated with actinomycin D for 24 h shown in Fig. 2*A* (*Lower*). The S-phase population was considerably reduced compared to the untreated cells shown above. Cells with the E6 and E7 proteins from the low-risk HPV6 also underwent growth arrest after DNA damage and behaved like vector-infected cells in this assay. As previously shown (27), cells expressing 16E6 that had degraded the p53 protein did not undergo growth arrest. Cells with 16E7 also did not growth arrest after actinomycin D treatment.

Depletion of the S-phase population as a result of actinomycin D-induced DNA damage was quantitated from cells staining FITC (BrdUrd) positive and is shown in graphic form in Fig. 2*B*. Data from three to five independent infections of HFEs derived from different sources are shown. Cells infected with LXSN, 6E6, or 6E7 had only 3–30% of the number of cells in the S-phase population after actinomycin D treatment compared to untreated cells. Cells expressing the *16E7* gene alone retained 60–90% of their S-phase population after treatment and were only slightly less effective at abrogating the block as completely as cells expressing the *16E6* gene, either alone or in combination with *16E7*, which had nearly the same number of cells in the S-phase population after treatment. Table 1 shows the average population in each

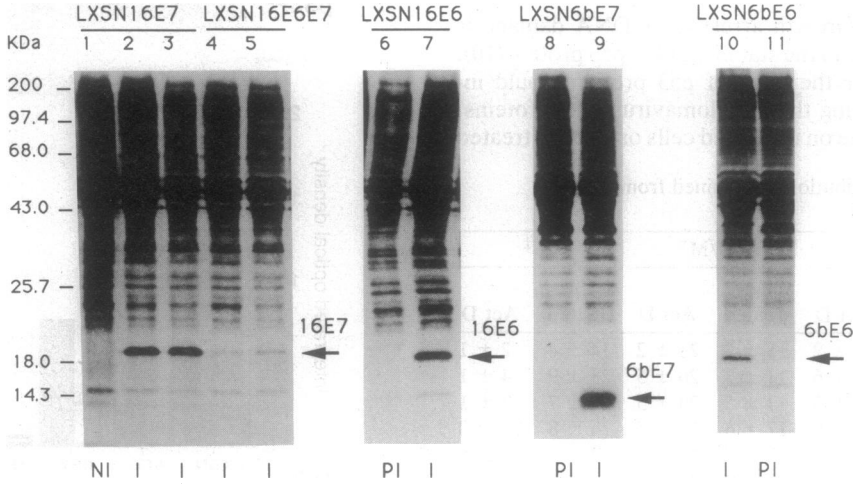

FIG. 1. Radioimmunoprecipitation of papillomavirus proteins early after infection of primary human keratinocytes with retroviruses. Cells were metabolically labeled in medium containing [^{35}S]cysteine and [^{35}S]methionine at one passage after G418 selection. Extracts from cells infected with retroviruses carrying 16E7 (lanes 1–3), 16E6E7 (lanes 4 and 5), 16E6 (lanes 6 and 7), 6E7 (lanes 8 and 9), and 6E6 (lanes 10 and 11) genes were incubated with preimmune serum (lanes 1, 6, 8, and 11) or immune serum against 16E7 (lanes 2–5), 16E6 (lane 7), 6E7 (lane 9), or 6E6 (lane 10). Arrows point to the respective HPV proteins.

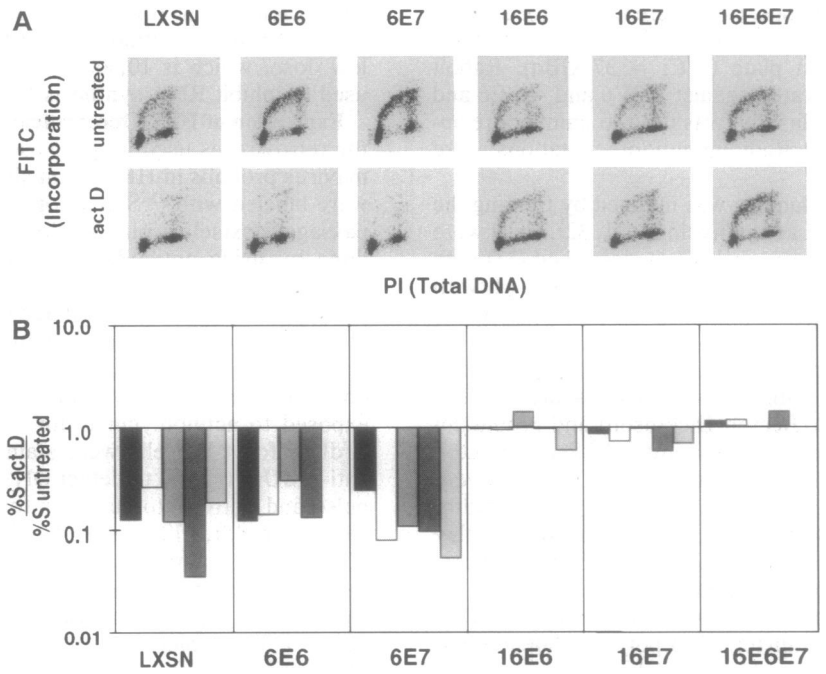

FIG. 2. Flow cytometric analysis of cells untreated and treated with actinomycin D (act D). (*A*) Data are presented as a two-parameter plot FITC (DNA incorporation) vs. PI (total DNA) staining. (*Upper*) Untreated cells. (*Lower*) Actinomycin D-treated cells. (*B*) Ratio of percentage S-phase population treated to percentage S-phase population untreated was plotted. Each bar represents data from an independent retroviral infection into epithelial cells derived from different foreskin samples. S-phase population was determined from plots such as those shown in *A* as percentage cells staining positive for BrdUrd.

phase of the cell cycle comparing untreated and DNA-damaged cells. After actinomycin D treatment, LXSN-, 6E6-, and 6E7-expressing cells accumulated in both G_1 and G_2. In some experiments, a population of cells existed that had S-phase DNA content but apparently did not incorporate BrdUrd (see Fig. 2*A*, especially 16E6, 16E7, and 16E6E7). These cells may have exited the cell cycle in S phase; however, there is no apparent effect from the actinomycin treatment on this population. In determining the cell cycle distribution from histograms the FITC-negative populations were split between the 2*n* and 4*n* peaks; therefore, these cells were included in the G_1/G_0 or G_2/M populations. Cells expressing 16E6, 16E7, and 16E6E7 do not block in G_1. Therefore, either 16E6 or 16E7 can abrogate the G_1 arrest after DNA damage.

p53 Protein Levels. Growth arrest after DNA damage is preceded by an increase in the stability of the p53 protein (10). To investigate whether the level of p53 protein would increase in cells expressing the papillomavirus oncoproteins, Western blots were done on untreated cells or on cells treated with actinomycin D for 24 h. Fig. 3 shows that the p53 protein levels were increased 2- to 10-fold upon actinomycin D treatment in all cells, except those expressing 16E6. The levels of p53 were extremely low in cells expressing 16E6 and longer exposures did not reveal any increase in p53 protein after exposure to actinomycin. We had previously noted that p53 protein levels were elevated in cells expressing the 16E7 oncoprotein (35), and in this experiment a 2- to 5-fold increase in p53 protein levels was observed in cells expressing 16E7

Table 1. Cell cycle distribution determined from two-parameter histograms

		G_1/G_0*		G_2/M†		S‡	
	n	Untreated	Act D	Untreated	Act D	Untreated	Act D
LXSN	5	70 ± 5	77 ± 2	15 ± 2	21 ± 2	14 ± 4	2 ± 1
6E6	4	63 ± 9	71 ± 6	14 ± 3	26 ± 5	23 ± 9	4 ± 1
6E7	5	66 ± 4	78 ± 5	13 ± 5	20 ± 5	21 ± 7	2 ± 1
16E6	5	58 ± 8	58 ± 8	17 ± 6	19 ± 5	24 ± 8	23 ± 8
16E7	5	60 ± 2	59 ± 5	18 ± 5	24 ± 6	22 ± 5	17 ± 5
16E6E7	3	56 ± 6	49 ± 6	20 ± 2	22 ± 1	24 ± 6	30 ± 6

Percentage cells ± SD is given for each phase of the cell cycle. Act D, actinomycin D.
*G_1/G_0 cells, BrdUrd negative with DNA content < 3*n*.
†G_2/M cells, BrdUrd negative with DNA content > 3*n*.
‡S cells, BrdUrd positive.

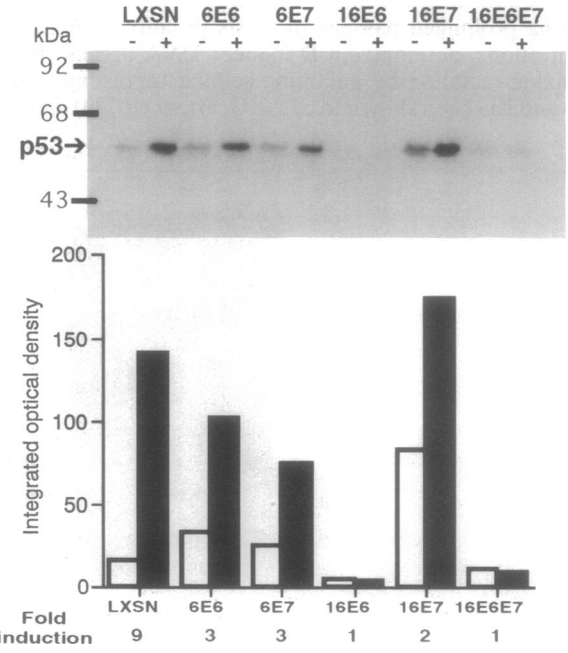

FIG. 3. (*Upper*) Western blot for p53 protein levels. Cell lysates were analyzed for p53 protein levels from untreated (lanes −) and actinomycin D-treated (lanes +) cells. (*Lower*) p53 levels in graphic format. □, Untreated; ■, actinomycin D treated.

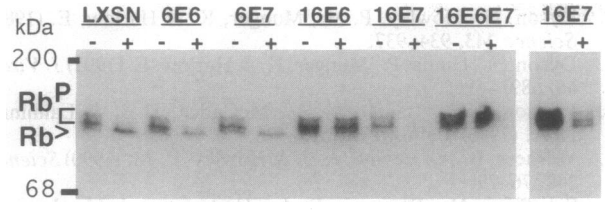

FIG. 4. Western blot for Rb protein levels and phosphorylation state. Cell lysates were analyzed for Rb protein from untreated (lanes −) and actinomycin D-treated (lanes +) cells. A longer exposure of the 16E7 lanes is shown at far right. Faster-migrating band is hypophosphorylated form of Rb. Slower-migrating bands are hyperphosphorylated forms (Rb^P).

compared to vector, 6E6, or 6E7 cells. A further 2-fold elevation in p53 protein was observed after treatment; thus, the ability to stabilize the p53 protein upon actinomycin D treatment was not impaired in the 16E7-expressing HFEs. Nonetheless, the cells did not growth arrest after DNA damage.

Rb Protein Levels and Phosphorylation. Since 16E7 was able to abrogate growth arrest and 16E7 associates with the Rb tumor suppressor protein (22), Western blot analysis of Rb protein in untreated cells and in cells after DNA damage was performed. The Rb tumor suppressor has multiple phosphorylated forms that run with various mobilities on SDS/PAGE (36). The fastest-migrating form is the hypophosphorylated form that appears in the cell after mitosis and becomes phosphorylated at the transit from G_1 to S. It is the underphosphorylated form that is thought to act in limiting cell proliferation and is the form that binds viral oncoproteins SV40 large tumor antigen and papillomavirus 16E7 (36, 37). Fig. 4 shows that the Rb protein levels dropped upon actinomycin D treatment, except in cells expressing 16E6. Rb levels had previously been shown to decrease due to type β transforming growth factor-induced growth arrest in epithelial cells (38). The Rb protein failed to become phosphorylated in all cells that underwent growth arrest—i.e., those cells containing LXSN, 6E6, or 6E7. A longer exposure of the lanes with 16E7 is shown at the far right and clearly demonstrates that the majority of Rb protein was not in the underphosphorylated form, in contrast to cells expressing LXSN, 6E6, and 6E7. In cells that abrogated G_1 arrest, the Rb protein became phosphorylated but the effect on the amount of Rb protein was different in cells expressing E6 vs. E7. Neither the levels nor the phosphorylation state of Rb protein changed in cells expressing 16E6 in response to DNA damage; in cells expressing 16E7 alone Rb protein levels dropped considerably. In cells expressing both 16E6 and 16E7, the Rb levels did not decrease after actinomycin D treatment, indicating that the effect on Rb of the *E6* gene was dominant over the *E7* gene.

DISCUSSION

We have shown that expression of either the *16E6* or the *16E7* gene in primary human epithelial cells was able to abrogate G_1 arrest after DNA damage. The oncoproteins from the low-risk HPV6 had no effect on growth arrest. It has been previously shown that 16E6 was able to disrupt the ability of p53 to modulate transcription (39, 40) and to arrest in G_1 after DNA damage (27). Because each of the small DNA tumor viruses produces oncoproteins that interact with the cellular tumor suppressors p53 and Rb, it has been suggested that cells have two independent pathways controlling cell proliferation and that both the p53 and Rb pathways must be inactivated in order to bring about the transformed phenotype. Expression of 16E7 in epithelial cells was able abrogate growth arrest, which has been shown to be mediated by p53; thus, the p53 and Rb pathways may be interconnected for at least some aspects of growth control. Three lines of evidence suggest this: first, cells expressing 16E7 have elevated p53 levels; second, in normal cells p53-mediated growth arrest changes the phosphorylation pattern of Rb, and 16E6 degradation of p53 alleviates the changes in Rb phosphorylation; third, 16E7 interacts with Rb and blocks growth arrest. The adenovirus *E1a* gene has been shown to cause p53 protein levels to increase (41) and to abrogate growth arrest after irradiation in rodent cells (33); therefore, another viral oncogene that interacts with Rb can interfere with this pathway.

We propose a model in which the growth arrest pathway after DNA damage that is mediated by p53 must involve the Rb protein. After DNA damage, p53 is activated and thereby becomes stabilized. p53 then would act as a transcriptional activator or repressor whose downstream targets lead to the inhibition of phosphorylation of Rb. In cells expressing 16E6, p53 is not present at a high enough level to modulate the downstream events and so this checkpoint control is bypassed. On the other hand, in cells expressing 16E7, p53 becomes stabilized and Rb protein levels drop as in vector-infected cells. The decrease in Rb levels may be a result of p53 acting to negatively regulate the Rb promoter (40). This is perhaps an indication that the growth arrest signal in cells with 16E7 has been transduced via p53. However, as opposed to cells that undergo growth arrest, the lower level of Rb protein does not remain underphosphorylated, but in the presence of 16E7 the inhibition of Rb phosphorylation is somehow relieved.

The hypothesis that Rb and p53 pathways are interconnected has been supported by the recent publication of papers that describe the cloning of a p21 gene variously named *WAF1*, *CIP1*, and *SDI1* (42–45). The p21 gene is transcriptionally up-regulated by p53. Furthermore, the p21 protein binds to and inhibits cyclin/cdk complexes, including those that can phosphorylate Rb. Thus, p21 ties the p53 and Rb pathways in a single step.

Growth arrest signals set up a series of events in which phosphorylation of Rb is inhibited at the G_1 exit. It has been proposed that viral oncoproteins that inactivate Rb do so by binding to underphosphorylated Rb, thereby releasing free E2F (46). Either after type β transforming growth factor treatment of cells expressing SV40 large tumor antigen (38) or after DNA damage of cells expressing 16E7 Rb does not remain hypophosphorylated. There are two possible explanations for the observation of phosphorylated Rb. One is that the free E2F has driven the cell into S phase and as a result Rb has become phosphorylated. Another is that the Rb must become phosphorylated for the cell to pass through the restriction point. The second model implies that in addition to binding Rb, viral oncoproteins may supply further activities in order to inactivate the antiproliferative properties of tumor suppressor proteins.

E1a proteins with mutations that result in no binding to Rb were still able to induce phosphorylation of Rb in quiescent BRK cells (47). Concomitant with Rb phosphorylation was cellular DNA synthesis. This implied that Rb can become phosphorylated in the absence of direct binding to E1a. Perhaps the viral oncoproteins act as a nucleus to reconstitute an active cyclin kinase complex that allows the cells to transit through the cell cycle at points where the cell is receiving growth arrest signals.

Each of the DNA tumor virus proteins has now been observed to allow the phosphorylation of Rb under conditions in which Rb is normally underphosphorylated. This implies that the mere binding of Rb is not the only mechanism by which the viral oncoproteins can exert their effect. Binding to Rb may play a role in the action of the viral oncoproteins but this may not be the sole mechanism of action. Understanding how these viral oncoproteins are able to

circumvent the normal cell regulatory systems will contribute to understanding the regulation of the cell cycle.

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